Attorney Docket No. 12879/2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Confirmation No.: 6940

INVENTORS	:	Gautam Vinod Daftary et al.
APPLICATION NO.	:	10/748,094
FILED	:	December 31, 2003
FOR	:	NON-PEGYLATED LONG-CIRCULATING LIPOSOMES
EXAMINER	:	Kishore, Gollamudi S.
ART GROUP	:	1615

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUBMISSION OF PRIORITY DOCUMENT

Sir:

Submitted herewith is a certified copy of the priority document, Indian Application No. 1101/MUM/2002, for the above-identified application.

Respectfully submitted,
KENYON & KENYON LLP

Date: April 18, 2007

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Government Of India
Patent Office
Todi Estates, 3rd Floor,
Lower Parel (West)
Mumbai – 400 013

THE PATENTS ACT, 1970

IT IS HEREBY CERTIFIED THAT, the annex is a true copy of Application and Provisional Specification filed on 9/12/2002 and post dated to 31/12/2002 under section 17(1) in respect of Patent Application No. 1101/MUM/2002 of Bharat Serums & Vaccines Ltd., Road No. 27, Wagle Estate, Thane-400 604, Maharashtra, India, an Indian company incorporated under the Companies Act, 1956.

This certificate is issued under the powers vested in me under Section 147 (1) of the Patents Act, 1970.

Dated this 1st day of March 2004.

(N. K. GARG)
ASST. CONTROLLER OF PATENTS & DESIGNS

FORM 1

THE PATENTS ACT, 1970 (39 of 1970)
APPLICATION FOR GRANT OF A PATENT
[See sections 5(2), 7]

1. **We, Bharat serums & Vaccines Ltd., Road No. 27, Wagle Estate, Thane – 400 604. Maharashtra, India.**
an Indian company incorporated under the Companies Act 1956,

2. hereby declare
   a) that we are in possession of an invention titled **" A process for the manufacture of low toxicity Non-pegylated antitumor liposomes."**
   b) that the **provisional specification** relating to this invention is filed with this application.
   c) that there is no lawful ground of objection to the grant of a patent to us.

3. We further declare that the inventors for the said invention are
   a) **Dr. Daftary Gautam Vinod**
   Bharat Serums & Vaccines Ltd., Road No. 27, Wagle Estate
   Thane – 400 604., Maharashtra, India
   Nationality - Indian
   b) **Mr. Pai Srikanth Annappa**
   Bharat Serums & Vaccines Ltd., Road No. 27, Wagle Estate
   Thane – 400 604., Maharashtra, India
   Nationality - Indian
   c) **Ms. Rivankar Sangeeta Hanurmesh**
   Bharat Serums & Vaccines Ltd., Road No. 27, Wagle Estate
   Thane – 400 604., Maharashtra, India.
   Nationality - Indian

ORIGINAL

1101 | मुंबई MUM | 2002

- 9 DEC 2002

The application is post dated to 31.12.2002 u/s 17(1) of the Patents Act, 1970 (as amended in 2002)

4. We claim the priority from the application filed in convention countries. particulars of which are as follows - **None**

5. We state that the said invention is an improvement in or modification of - **Not Applicable**

6. We state that the application is divided out of our application. the particulars of which are given below and pray that this application be deemed to have been filed on _____ under section 16 of the Act. - **Not Applicable**

Examiner of Patents & Designs

7. That we are the assignee or legal representative of the true and first inventors.

1101/mum/2002

9/12/2002

8. That our address for service in India is as follows :

C/o Mr. Kasbekar Madhav Gajanan **Tel. : 4305030**
48 / 3, Madhavi Sah-Nivas, 277, Mogul Lane,
Mahim, Mumbai – 400 016., Maharashtra, India.

9. Following declaration was given by the inventors.

We, the true and first inventors for this invention declare that the applicant herein is our assignee.

a) ..... **Dt.** 3/12/2002
DR. DAFTARY GAUTAM VINOD

b) ..... **Dt.** 3/12/02
PAI SRIKANTH ANNAPPA

c) ..... **Dt.** 3/12/2002
RIVANKAR SANGEETA HANURMESH

10. That to the best of our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to us on this application.

11. Following are the attachments with the application.
a) Provisional specification - 3 copies
b) Fee Rs. 5,000.
c) Statement and undertaking on Form 3.
d) Power of Attorney

We request that a patent may be granted to us for the said invention.

Dated this 3rd day of December 2002.

For **BHARAT SERUMS & VACCINES LTD.**

DR. DAFTARY GAUTAM VINOD
Director

To
The Controller of Patents
The Patent Office
Mumbai.

FORM 2

THE PATENTS ACT, 1970
PROVISIONAL SPECIFICATION
[See section 10]

ORIGINAL

Title : ***"A process for the manufacture of low toxicity Non-pegylated antitumour liposomes"***

Applicant : ***BHARAT SERUMS & VACCINES LTD.,***
Road No. 27, Wagle Estate,
Thane – 400 604. Maharashtra, India.

an Indian company incorporated under the Companies Act 1956,

The following specification describes the nature of the invention

A process for the manufacture of low toxicity Non-pegylated parenteral compositions comprising antitumor liposomes.

Field of Invention :

This invention relates to a process in the field of manufacture of low toxicity Non-pegylated antitumor liposomal compositions. This invention particularly relates to the process for the manufacture of Non-pegylated, long circulating, sterile, low toxicity, antitumor liposomal compositions comprising Anthracycline antineoplastic agents.

Background and prior art :

Liposomes are composed of phospholipids and / or cholesterol and consist of vesicular structure based on lipid bilayers surrounding aqueous compartments. They vary widely in their physicochemical properties such as size, surface charge, phospholipid composition in the type of phospholipids used. Conventional liposomes are characterised by a relatively shorter circulation time when administered intravenously and also they tend to accumulate rapidly in the reticuloendothelial system. These conventional liposomes were not effective in delivering the drugs in required concentrations at tumor site. This led to the development of long-circulating liposomes which are able to extravasate to tumor site which are highly vascular in nature. Long circulating liposomes are prepared by using pegylated phospholipids in the phospholipid composition. These pegylated liposomes are also called sterically stabilized liposomes or Stealth liposomes. This liposomes have got the ability to localise preferentially in tumors by the very nature of their long circulating time. The other advantage with this type of liposomes is the limited availability of the drug in the tissues of sensitive organs thereby reducing the toxicity. This changed biodistribution has also led to appearance of new toxic effects. The main disadvantage of using long circulating liposomes containing Pegylated phospholipids is the Hand-foot syndrome reported in clinical uses wherein skin eruptions have been observed on the palms of the hands and soles of the feet.

Though ideally anticancer drugs have to be specifically toxic to tumor cells, most of the anticancer drugs are toxic to the normal cells as well. Most of the drugs used in cancer chemotherapy have a narrow therapeutic index and result in undesirable side effects leading to patient non-compliance and suboptimal dosage. Doxorubicin hydrochloride (DXR) is one of the most commonly used cytotoxic Anthracycline antibiotic used in cancer chemotherapy. It has activity against a wide variety of human neoplasms. Alberto A Gabizon has reviewed toxicity of Doxorubicin hydrochloride. This drug has different type of toxicity such as cardiac toxicity, anaphylactic reaction, emetogenicity, myelosuppression, mucocytis, skin toxicity, alopecia and toxicity to injection sight (Ref.:Cancer Investigation, 19(4): 424-436(2001).

In order to reduce the toxicity, deliver the drug at the tumor site and thus increase its therapeutic activity Doxorubicin hydrochloride was entrapped in liposomes. The liposome vesicles are prepared using phospholipids and cholesterol and then they are loaded with the drug, by driving the anionic drug through the vesicle bilayer membrane by creating pH gradient or chemical gradient across the membrane, into the vesicle.

US Patent 4769250 (Sept. 88) describes making of liposomes containing Anthracycline antineoplastic agents with anionic and neutral phospholipids. The particles are in the form of vesicles which comprise Daunorubicin, distearoyl phosphatidylglycerol, and distearoyl phosphatidylcholine. The mole ratio of Daunorubicin to distearoyl phosphatidylglycerol is at least about 1:1.25, and the suspending medium is a low ionic strength aqueous lactose solution containing a small amount of base.

However, intravenously administered liposomes are taken up by the cells of the mononuclear phagocytic system immediately after administration. Rapid clearance of liposomes is due to the uptake by the immune system as well as disintegration resulting from electrostatic, hydrophobic and Vander Waals interactions of liposomes with plasma protein. Electrostatic and hydrophobic interactions can be minimised by using neutral lipids and mechanically very strong bilayers. Number of patents, mainly US 4501728, US 4920016, US 5013556 and US 6132763 describe liposomal preparations with enhanced circulation time.

US Patent **4501728** describes masking of liposomes from reticuloendothelial system (RES) recognition. A biochemical membrane covered with sialic residues thereby provides a coating that masks the surface membrane from recognition and removal by the scavenging RES cells of the body.

US Patent 4920016 describes liposomes with enhanced circulation time. The composition of liposomes essentially contain an entrapped pharmaceutical agent such as Bleomycin, Doxorubicin and are characterized by liposome sizes predominantly between about 0.07 and 0.5μ, contains at least 50mole% of a membrane rigidifying agent lipid such as sphingomyelin or neutral phospholipids with predominantly saturated acyl chains, contains about 5 to 20 mole% of a glycolipid selected from the group consisting of ganglioside, saturated phosphatidylinositol and monogalactosyl stearate. These liposomes show high blood / RES tissue distribution ratios, and are effective for drug administration to tumors via intravenous drug delivery.

US Patent 5185154 describes a method for instant preparation of large unilamellar vesicles of uniform size distribution containing lipid soluble pharmaceutical agent such as Beclomethasone propionate, hydrocortisone.

US Patent 5013556 describes liposomes with enhanced circulation time containing amphipathic antitumor compound such as Doxorubicin hydrochloride. A liposome composition containing amphipathic lipid derivatized with a polyalkylether, as exemplified by phosphatidylethanolamine derivatized with polyethylene glycol. The derivatized lipid enhances the circulation time of the liposomes several fold. This enhancement is achieved by either fluid or membrane rigidifying liposome components.

US Patent 6132763 describes liposomes with covalently bound PEG moieties on the external surface which demonstrate improved serum half-life following intravenous administration. The PEG moieties are linked to amino groups in the head group of at least one phospholipid species forming the liposomes. Suitable phospholipids having amino groups in the head group include phosphatidylethanolamine and phosphatidyl serine. This pegylated phospholipid when incorporated along with other phospholipids brings about

stearic stabilisation wherein Vander Waals attraction is reduced thereby increasing blood circulation half-lives. (Danilo D Lasic, Nature, vol.380, 11 April 1996, pg 561). These liposomal preparations containing pegylated phospholipids however lead to skin toxicity generally known as Hand-foot Syndrome. [Ref. Kenneth B Gordon, Cancer, Vol. 75(8), 1995, pg 2169 - 2173]

US Patent 6083530 describes a three component systems which are required to be mixed at the time of administration. This liposomal preparations have preferably a high drug lipid ratio. Active ingredient is loaded by an active mechanism using a transmembrane ion gradient. Drug lipid ratios employed are about 3 - 80 fold higher than for traditional liposome preparations. The lipid comprises at least one phospholipid selected from the group consisting of Egg phosphatidylcholine (EPC), distearoyl phosphatidylcholine (DSPC), Hydrogenated soya phosphatidylcholine (HSPC), Dipalmitoyl phosphatidylcholine (DPPC), Dimyristoyl phosphatidylcholine (DMPC) and Diarachidonoyl phosphatidylcholine (DAPC). The three-compartment system comprises an ionizable basic antineoplastic agent, a release inhibiting aqueous buffer comprising Citric acid and a bilayer comprising a phospholipid.

Another problem of Doxorubicin hydrochloride liposomes is the leakage of encapsulated drug. To some extent this leakage has been reduced by incorporation of cholesterol in the liposomes. [Alberto A Gabizon, Cancer Investigation, 19(4) 424-436(2001)].

European Patent 0655 239 (Aug. 99) describes use of niacin, vitamin E analogs in liposomal compositions containing antineoplastic agents such as Doxorubicin hydrochloride.

Object of Invention :

The main object of the present invention is to improve the process for manufacture of a parenteral composition comprising low toxicity, sterile Anthracycline antineoplastic liposomes.

Another object of present invention is to increase the circulation time without using the pegylated phospholipids so that the drug is made effective as measured by in-vivo efficacy studies in animals.

Accordingly, the present invention relates to a process for manufacture of a long circulating, low toxicity, sterile Non-pegylated liposomes essentially comprising Anthracycline antineoplastic agents, one or more phospholipids, cholesterol; the process for manufacture comprising,

(i) dissolving one or more phospholipids, cholesterol in a single solvent or in a mixture of solvents, and then removing the solvents by evaporation under reduced pressure to form a dry lipid film;

(ii) hydrating the thin lipid film with an aqueous buffer to form liposomes;

(iii) sizing the liposomes to the desired size;

(iv) removing the free buffer salts from the solution containing liposomes by dialysis or ultrafiltration or column chromatography;

(v) loading the liposomes with the drug (Anthracycline antineoplastic agents);

(vi) removing the unentrapped drug;

(vii) making up the volume to have the desired content of the drug in the bulk solution;

(viii) aseptically filtering the bulk through 0.2μ filter, filling the filtrate into sterile depyrogenated container, sealing the container under nitrogen cover to obtain the product suitable for parenteral administration.

Description of the invention :

In one embodiment of the invention, the Anthracycline antineoplastic agent used is Doxorubicin hydrochloride.

The content of Doxorubicin hydrochloride in the composition of present invention varies from 1mg/ml to 5mg/ml of the composition, preferably the content of Doxorubicin hydrochloride is 2mg/ml of the composition.

The total content of phospholipids varies from 4mg/ml to 30mg/ml of the composition. The preferred content is from about 8mg/ml to about 16mg/ml of the composition.

The weight ratio of Doxorubicin hydrochloride to phospholipids is from about 1:2 to about 1:15. The preferred weight ratio is from about 1:4 to about 1:10

In the process of present invention, phospholipids are preferably chosen from Dimyristoyl phosphatidylcholine (DMPC), Dimyristoyl phosphatidylglycerol sodium salt (DMPG), Dipalmitoyl phosphatidylcholine (DPPC), Dipalmitoyl phosphatidylglycerol (DPPG), Distearoyl phosphatidylcholine (DSPC), Distearoyl phosphatidylglycerol (DSPG), Distearoyl phosphatidylethanolamine (DSPE) etc. or other derivatised phospholipids. The preferred phospholipid is Distearoyl phosphatidylcholine (DSPC).

Examples :

The invention will now be illustrated by way of Examples. The Examples are by way of illustration only and in no way restrict the scope of the invention.

All the raw materials used in these Examples were of parenteral grade. Equipments used were of conventional nature. Entire processing was done in an area with a controlled environment required for manufacturing sterile products.

Doxorubicin hydrochloride used in these Examples was of parenteral grade complying with Indian Pharmacopoeial specifications.

Phospholipids DMPC, DMPG, DSPC, DSPG, DPPC, DPPG were obtained from Lipoids.

Organic solvents used in the Examples were of AR (Analytical reagent) quality.

Example 1 :

Process of making liposomal composition containing Doxorubicin hydrochloride

Lipid film formation : DSPC (1.565gm) and Cholesterol (0.521gm) were dissolved one after the other in Chloroform (40ml) taken in a Rotary evaporator flask. A thin film of lipids was formed by evaporating Chloroform under vacuum at 60°C. After releasing the vacuum, the flask was rotated for 15 minutes while passing nitrogen into the flask to dry off any residual solvent.

Hydration : Lipid film was hydrated with 60ml of aqueous buffer solution containing Ammonium sulfate. The liposomal suspension obtained was sized by extruding successively through filters having pore size from 0.4μ and upto 0.05μ.

Dialysis : Suspension of blank sized liposomes was dialysed using Histidine buffer to create gradient. The dialysis buffer was changed repeatedly.

Drug loading : Doxorubicin hydrochloride (216mg) was dissolved in 14ml of Histidine buffer and added to 40ml of blank sized liposomes and mixed for 1 hour. Drug loaded liposomal dispersion was then passed through a Dowex column to remove unentrapped drug.

The product obtained after passing through the Dowex column had the following characteristics.

Product analysis

Total Doxorubicin HCl content	3.98mg/ml
Entrapped Doxorubicin HCl content	3.94mg/ml

The above product after dilution with Histidine buffer to a concentration of 2mg/ml was analysed for the following parameters:

Appearance : Red coloured translucent liquid.

pH : 6.1

Particle size	:	Average particle size 92.7nm with less than 10% of particles below 105.4nm.
Doxorubicin HCl content	:	2.01mg/ml.
Bacterial endotoxins	:	Less than 5 EU/mg of Doxorubicin hydrochloride.
Sterility	:	Sterile

This composition was subjected to Acute toxicity studies in mice and the LD_{50} dose was found to be 16mg/kg whereas the LD_{50} dose for the marketed conventional preparation was 10mg/kg and for the marketed liposomal preparation it was 12mg/kg.

Example II :

Procedure same as Example I except that instead of using 60ml, 120ml of hydration buffer was used.

Drug loading : Doxorubicin hydrochloride (180mg) was dissolved in 10ml of Histidine buffer and added to 80ml of blank sized liposomes and mixed for 1 hour. Drug loaded liposomal dispersion was then passed through a Dowex column to remove unentrapped drug.

The product obtained after passing through the Dowex column had the following characteristics.

Appearance	:	Red coloured translucent liquid.
pH	:	6.1
Particle size	:	Average particle size 104.4nm with less than 10% of particles below 120nm.
Doxorubicin HCl content	:	1.98mg/ml.
Bacterial endotoxins	:	Less than 5 EU/mg of Doxorubicin hydrochloride.
Sterility	:	Sterile

Using the composition of Example II Acute toxicity, Pharmacokinetic studies in healthy mice and Efficacy study on MCF-7, and L1210 cell-lines in nude mice

ACUTE TOXICITY AND PHARMACOKINETIC STUDIES IN MICE

Parameters	Example II	Caelyx*
LD_{50} (mg/kg)	16.13	13.5
MTD (mg/kg)	8	8
C_{max} (mcg/ml)	267.54	285.74
T_{max} (hours)	0.085	0.085
Kel	0.0997	0.07109
$T_{1/2}$ (hours)	6.948	9.748
AUC (mcg-hr/ml)	1694.024	2083.215
Vd (ml)	1.480	1.688
Vd (ml/kg)	59.20	67.52
Cl (ml/hr)	0.15	0.12

* Marketed formulation

In-vivo efficacy study on MCF-7 and L1210 Cell-lines indicated better efficacy of the product made by the process of present invention.

EFFECT ON MCF-7 HUMAN BREAST TUMOR IMPLANTED IN NUDE MICE

Group	Average Tumor Weight (mg)				
Day	Saline Control	Example II (6mg/kg)	Example II (12mg/kg)	Caelyx* (6mg/kg)	Caelyx* (12mg/kg)
1	36.5	31.5	68.4	44.3	57.8
5	49.5	45.3	81.6	59.5	50.7
10	63.1	40	43.6	52.2	31.3
15	54.3	42.8	46.1	28.5	32
20	78.12	5.3	16.2	26.6	27.8
25	105	3	8	19.8	22.8
30	95.4	3	8	22.12	16.6
Wt.	58.9	-28.5	-60.4	-22.18	-41.2
T/C%	NA	-90.47	-88.3	-50	-71.2

* Marketed formulation

ANTI-TUMOR ACTIVITY AGAINST L1210 MOUSE LEUKEMIA MODEL

Group	Dosage (mg/kg)	Mice	Survival Time (Days)	Mean Survival Time (Days)	T/C%
Saline Control	NA	1/5	17	16	NA
		2/5	16		
		3/5	17		
		4/5	16		
		5/5	16		
Example II	6	1/5	20	20.4	128
		2/5	20		
		3/5	22		
		4/5	20		
		5/5	20		
Example II	12	1/5	23	21.2	132
		2/5	20		
		3/5	20		
		4/5	20		
		5/5	23		
Caelyx*	6	1/5	18	20.4	128
		2/5	22		
		3/5	20		
		4/5	20		
		5/5	22		
Caelyx*	12	1/5	18	20.6	129
		2/5	22		
		3/5	20		
		4/5	23		
		5/5	20		

* Marketed formulation.

Advantages of the invention:

1. Low toxicity profile
2. Longer circulation time
3. Proven efficacy with antitumor activity on MCF-7 and L1210 Cell-lines
4. Free from Hand-Foot-Syndrome

Dated this 3rd day of December 2002.

For **BHARAT SERUMS & VACCINES LTD.**

DR. DAFTARY GAUTAM VINOD
Director

To,
The Controller of Patents
The Patent Office
Mumbai.